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# **Periapical fluid RANKL and IL-8 are differentially regulated in pulpitis and apical periodontitis**

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**Running title:** RANKL and IL-8 in periapical disease

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## **Abstract**

The dental pulp space can become infected due to a breach in the surrounding hard tissues. This leads to inflammation of the pulp (pulpitis), soft tissue breakdown, and finally to bone loss around the root apex (apical periodontitis). The succession of the molecular events leading to apical periodontitis is currently not known. The main inflammatory mediator associated with neutrophil chemotaxis is interleukin-8 (IL-8), and with bone resorption the dyad of receptor activator of NF- $\kappa$ B ligand (RANKL) and osteoprotegerin (OPG). The levels of RANKL, OPG and IL-8 were studied in periapical tissue fluid of human teeth (n=48) diagnosed with symptomatic irreversible pulpitis (SIP) and asymptomatic apical periodontitis (AAP). SIP represents the starting point, and AAP an established steady state of the disease. Periapical tissue fluid samples were collected using paper points and then evaluated using enzyme-linked immunosorbent assays (ELISAs). Target protein levels per case were calibrated against the corresponding total protein content, as determined fluorometrically. RANKL was expressed at significantly higher levels in SIP compared to AAP ( $P<0.05$ ), whereas OPG was under the detection limit in most samples. In contrast, IL-8 levels were significantly lower in SIP compared to AAP ( $P<0.05$ ). Spearman's correlation analysis between RANKL and IL-8 revealed a significantly ( $P<0.05$ ) negative correlation between the two measures ( $\rho = -.44$ ). The results of this study suggest that, in the development of apical periodontitis, periapical bone resorption signaling, as determined by RANKL, occurs prior to inflammatory cell recruitment signaling, as determined by IL-8.

**Keywords:** RANKL, OPG, interleukin-8, biomarkers, apical periodontitis.

*Abbreviations:* IL, interleukin; RANKL, receptor activator of NF- $\kappa$ B ligand; OPG, osteoprotegerin; SIP, symptomatic irreversible pulpitis; AAP, asymptomatic apical periodontitis

## 1. Introduction

The oral cavity is a unique environment, where hard tissue structures (teeth) breach a soft tissue barrier into a space replete with microorganisms. Consequently, when the microorganisms manage to overcome this barrier, an opportunistic infection can occur. Examples of opportunistic oral infections include caries, periodontitis, and apical periodontitis (1). Apical periodontitis is fairly common in adults, with roughly one in three individuals affected (2). As any other opportunistic infection, apical periodontitis is characterized by a complex interplay between microbial tissue invasion and host defense (3). In the course of pulpal inflammation, the soft tissue inside the root canal system is digested by proteolytic enzymes, which are produced by neutrophils (4, 5). Unless the entryway for the microorganisms into the pulp space is blocked by intervention (6), the whole canal system can gradually become infected, and an inflammatory lesion establishes around the apical region to keep this infection under control (7). One histopathologic endpoint of periapical inflammation is bone loss, which may occur to increase vascularization at the portals of the apex, thus blocking the infection in the root canal space from affecting the host (7, 8).

The primary line of defense against microorganisms is the innate immune system. Pulpal and later periapical inflammatory responses are characterized by the influx of neutrophils (7). The main molecules associated with neutrophil recruitment and activation to the site of infection is interleukin-8 (IL-8, CXCL8) (3). IL-8 is produced at local sites of inflammation and has been associated with pulpal breakdown and apical periodontitis (9, 10). Periapical bone loss is a hallmark of apical periodontitis. On the molecular level, bone resorption is orchestrated by the interplay of receptor activator of NF- $\kappa$ B ligand (RANKL) and osteoprotegerin (OPG). The presence of RANKL activates its cognate RANK receptor and stimulates differentiation of precursor cells into osteoclasts. On the contrary, the OPG soluble decoy receptor blocks RANKL,

hence preventing its interaction with RANK, and therefore inhibits osteoclast activation and subsequently bone resorption (11, 12). The RANKL-OPG system is clearly involved in the pathogenesis of periodontal disease, and its relative RANKL/OPG ratio can indicate the occurrence, yet not the progression, of the disease (13). Since apical periodontitis is also characterized by bone loss, it is fair to postulate a role of this bi-molecular system in the pathogenesis of this disease as well. Indeed, a recent comprehensive review has summarized the information available of the RANKL-OPG system in the context of pulpal and periapical disease, identifying a lack of conclusive information (14).

What is currently not known in the context of periapical inflammation and bone resorption, is the succession of the molecular events that culminate in bone resorption or inflammatory cell recruitment. It is hypothesized that the molecular activation of bone resorption in the periapical region, hence the regulation of RANKL and OPG, should precede the chemotactic signals that lead to inflammatory cell recruitment, as evaluated by the regulation of IL-8. Therefore, it was the aim of the current study to investigate the periapical tissue fluid levels of RANKL, OPG and IL-8 in teeth with inflammation restricted to the pulp space, and compare these to counterparts from teeth with an established periapical inflammatory lesion.

## **2. Materials and methods**

### **2.1 Patients, Operative Procedures and Periapical Fluid Collection**

This study was approved by the Ethics Review Board of the Canton of Zürich (KEK-ZH-No. 2011-0253/4) and was conducted in accordance with the guidelines of the World Medical Association Declaration of Helsinki. It is confirmed that this cross-sectional study conforms to STROBE guidelines for observational studies. All patients were treated at the Department of Preventive Dentistry, Periodontology and Cariology, University of Zürich, Center of Dental

Medicine, Switzerland by one operator specialized in endodontics (DKR). The participants were in need of a root canal treatment. They were either referred for the treatment or attended the department's emergency unit during service hours. The patients were asked to participate in the study when they were of full age ( $\geq 18$ y) and were excluded from the study if they: i) refused to participate in the study, ii) were under long-term anti-inflammatory medication, immunosuppressive chemotherapy or any antibiotic medication, iii) were not systemically healthy (i.e. suffer from cardiovascular and respiratory disease, diabetes mellitus, HIV infection or hepatitis), or iv) were pregnant or in lactation. The clinical condition was diagnosed according to the patient's case history, clinical inspection, palpation, tenderness to percussion, vitality testing, probing depth and radiographic examination (single-tooth radiograph, Digora, Soredex, Tuusula, Finland). The clinical conditions included in the study were symptomatic irreversible pulpitis (SIP) with normal apical tissues and pulp necrotic or previously treated teeth with asymptomatic apical periodontitis (AAP) as defined by the American Association of Endodontists (15). The patients who entered the study gave written informed consent. All operative procedures were performed using a dental microscope under suitable magnification. For root canal treatment the patient's teeth were anesthetized with 4% articain (Septanest, Septodont, Saint-Maur-des-Fossés Cedex, France) and isolated with rubber dam. The work field was wiped with a cotton pellet soaked with 3% sodium hypochlorite (Kantonsapotheke, Zürich, Switzerland) for surface disinfection. When caries was present, it was excavated with round burs and a pre-endodontic build-up placed if deemed appropriate. The endodontic access was prepared with a sterile diamond-coated bur and the root canals were instrumented using ProTaper instruments (Maillefer Dentsply, Ballaigues, Switzerland) according to the manufacturer's recommendations. Endodontic working length was determined endometrically (Root ZX mini, J Morita Corp., Tustin, CA, USA) with a hand file (Maillefer Dentsply). The diagnosed clinical

condition (SIP or AAP) was confirmed during instrumentation by assessing the content of the root canal for the presence or absence of vital tissue. The root canals were instrumented up to their apical constriction, under continuous manual irrigation with 1% sodium hypochlorite (Kantonsapotheke) using a syringe with a side-vented needle (Hawe Irrigation Probe, gauge no. 30, Kerr Hawe, Bioggio, Switzerland) one millimeter shorter than the predetermined working length. A size-15 hand file was frequently used to keep the apical foramen patent. After instrumentation to ProTaper F2, five mL of sterile physiological saline solution (.9%, B. Braun Medical AG, Sempach, Switzerland) were administered to full working length to inactivate possible remnants of NaOCl. After drying the root canal with sterile paper points a fine paper point (Orbis Dental, Münster, Germany) was inserted approximately 2 mm above the apical foramen to collect the periapical tissue fluid (16). The paper point was kept in that position for 30 sec and was then transferred into a sterile micro-centrifugation tube (Biopure 1.5 ml, Vaudaux-Eppendorf AG, Schönenbuch, Switzerland). Three paper points were collected from one canal and immediately after frozen at -80°C until further processing. Only one root canal per tooth/patient has been sampled as described above. In case of multi-rooted teeth diagnosed with AAP one root corresponding with the radiographic lesion was sampled whereas in cases of multi-rooted teeth diagnosed with SIP the sampled canal was chosen randomly. After the periapical fluid collection the root canal treatments were finished *lege artis*

## **2.2 Sample Preparation**

For the laboratory analysis, a protease inhibitor (complete mini EDTA free, Roche, Basel, Switzerland) was added to sterile phosphate buffered saline (PBS, pH 7.2) and the samples were re-eluted in 300 µl of this mixture. The micro-centrifugation tubes were placed for 5 h on a platform shaker at (2000 rpm) at 4°C. Afterwards, the tubes were vortexed for 30 sec and

thereafter centrifuged for 10 min at 5000 rpm. The supernatant was collected and transferred into new micro-centrifugation tubes before being processed on 96-well plates for subsequent enzyme-linked immunosorbent assay (ELISA) analyses, while the resulting pellet was stored.

### **2.3 ELISA Assays for RANKL, OPG and IL-8**

The total amount of RANKL and OPG in the periapical supernatant was determined by human-specific ELISA according to the manufacturer's instructions (total sRANKL ELISA and Osteoprotegerin ELISA kits, Immundiagnostik AG, Bensheim, Germany). The concentrations of IL-8 in the supernatant were evaluated using a commercially available ELISA kit (DY208, Duo Set Human CXCL8/IL-8 ELISA, R&D Systems, Abingdon, UK). Absorbance was measured at 450 nm using a microplate reader (Epoch, BioTek, Lucerne, Switzerland), with wavelength correction of 620 nm for RANKL and OPG, and 570 nm for IL-8. A standard curve was created by a four-parameter logistic (4-PL) equation, using known concentrations of the rhRANKL, rhOPG and rhIL-8 standards, all provided in the corresponding ELISA kits. The concentrations of RANKL, OPG and IL-8 in all periapical fluid supernatants were calculated against these standard curves. Additionally, the concentrations of these analytes were also calibrated against the total protein content in each sample. Total protein in the periapical supernatant was quantified using a Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA), as recommended by the manufacturer.

### **2.4 Statistics and Data Presentation**

To compare differences between clinical diagnosis groups regarding categorical variables (gender, tooth type, or jaw), the Chi-Square test was used. Normality of the data related to continuous variables was checked by the Shapiro-Wilk test. These data sets were skewed, and



therefore for comparison between groups, Mann-Whitney U test was used. Correlation between RANKL and IL-8 levels was evaluated by the Spearman's correlation analysis. Statistically significant difference was considered at the level of  $P < 0.05$ . Continuous variables are presented as medians and interquartile ranges (IQRs).

### **3. Results**

#### **3.1 Patient Demographics and Samples Evaluated**

Fifty-eight patients (n=58) were recruited between September 2011 and May 2013. Periapical tissue fluid samples were collected from all patients, as described above. Ten samples could not be further analyzed for the following reasons: i) problems with the sampling procedure (i.e. severe blood contamination or it was impossible to sufficiently reach the periapical area with the paper point, n=7), ii) association with pus (i.e. pus discharge from the root canal, n=3). The remaining 48 samples were distributed SIP (n=21) and AAP (n=27) among the different clinical diagnosis. The ratio between female and male patients was 29/19 and the patient's age ranged between 18.0 and 80.5 years. Twenty-eight of the samples included were from molar teeth, 12 from premolars and 8 collected from anterior teeth. Twenty-six teeth were located in the maxilla and the remaining 22 teeth in the mandible. With regards to categorical variables, there were no statistically significant differences between the diagnostic groups when compared regarding gender, tooth type, or tooth location ( $P > 0.05$ ). Age of patients was also similar between groups, with no significant difference ( $P > 0.05$ ).

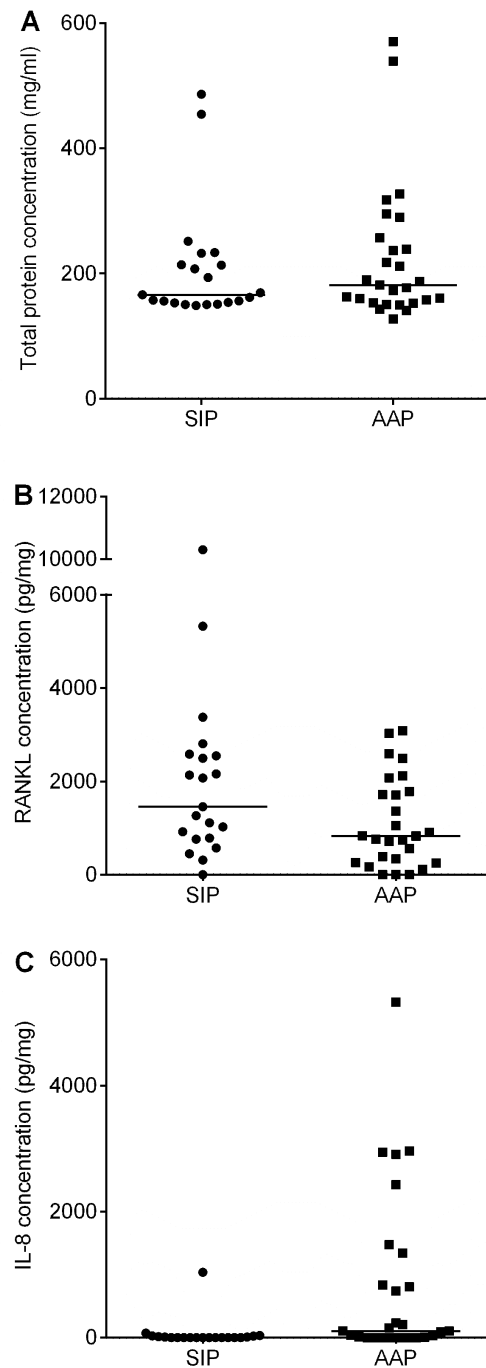
#### **3.2 Determination of RANKL, OPG and IL-8 Levels in Periapical Fluid**

The total protein concentration was first investigated, in order to evaluate if there were any quantitative differences in sampling amounts between the diagnostic groups. These were

(median, IQR) 166 (154, 223) mg/ml in SIP and 182 (154, 257) mg/ml in AAP, and the difference proved to be statistically non-significant (Figure 1A,  $P>0.05$ ). The levels of RANKL calibrated to total protein were further investigated. RANKL was detected in 20 of 21 samples in SIP (95 %) and in 24 of 27 samples with AAP (88 %). The median RANKL level for SIP was 1456 pg/mg total protein (IQR: 774 to 2567 pg/mg total protein). This was significantly ( $P<0.05$ ) higher in SIP than in AAP with 829 pg/mg total protein (IQR: 263 to 1785 pg/mg total protein; Figure 1B). On the other hand, OPG was barely detected in any of the periapical fluid samples, namely in 5 of 21 samples with SIP (24 %), and 3 of 27 samples with AAP (11 %). The low frequency of OPG detection prohibited any meaningful statistical analysis to determine differences between both groups. Moreover, due to the same reason, it was not possible to deduce the relative RANKL/OPG ratio in these samples.

The levels of IL-8 in the periapical fluid samples, calibrated to total protein, were investigated next. IL-8 was detected in 9 of 21 samples with SIP (43 %) and 20 of 27 samples with AAP (74 %). Periapical fluid samples of teeth with SIP exhibited significantly ( $P<0.05$ ) lower levels of IL-8 (median = not detected, IQR: not detected – 23 pg/mg protein) than in AAP (median = 106, IQR: not detected – 1340 pg/mg protein total protein; Figure 1C). Spearman's correlation analysis between the levels of RANKL and IL-8 in both conditions (values normalized to total protein) revealed a significantly ( $P<0.05$ ) negative correlation between the two measures ( $\rho = -.44$ ).

**Figure 1**



**Figure 1:** *Quantitative expression of total protein (A), RANKL (B) and IL-8 (C) in periapical tissue fluid samples of teeth diagnosed with symptomatic irreversible pulpitis (SIP) and asymptomatic apical periodontitis (AAP).* Total protein concentration (mg/ml) was determined fluorometrically (Qubit 2.0). Samples from SIP and AAP proved to be statistically similar

( $P>0.05$ ). RANKL concentration was determined by human-specific ELISA calibrated against the total protein content of each individual sample (pg/mg). RANKL levels were significantly higher in samples from SIP when compared to AAP ( $P<0.05$ ). IL-8 concentration was determined by human-specific ELISA calibrated against the total protein content of each individual sample (pg/mg). Periapical fluid samples of teeth with SIP exhibited significantly ( $P<0.05$ ) lower levels of IL-8 than AAP.

#### **4. Discussion**

In this study the periapical molecular inflammatory response to an opportunistic microbial infection was investigated. The findings demonstrate that at the starting point of apical periodontitis, as represented in SIP, where the inflammation is still confined to the root canal space, high levels of RANKL, but low levels of IL-8 are detectable in the periapical tissue fluid. In contrast, when an apical lesion is established and a chronic state of the inflammation has emerged, as is the case of AAP, significantly more IL-8, but less RANKL is present at the periapex. These findings strongly suggest, that in the kinetics of apical periodontitis, periapical bone resorption signaling, regulated by the RANKL/OPG system takes place prior to inflammatory cell recruitment mediated by IL-8, according to the hypothesis of the present study.

There are advantages employing apical periodontitis models for studying inflammatory responses such as a readily quantifiable endpoint of apical tissue destruction and the development of the pathology in a circumscribed area (8). However, in previous studies the kinetics of apical periodontitis has been investigated mainly employing rodent models where the pulp gets exposed to the oral environment (17). Pro-inflammatory cytokines, such as IL-1 and TNF- $\alpha$ , are expressed at a very early stage of the disease in rodent's periapical tissues (18). Especially IL-1 plays a pivotal role in development and progression of apical periodontitis (8). In addition, both IL-1 and

TNF- $\alpha$  are potent inducers of chemokine's further downstream such as IL-8. Nevertheless, the role of IL-8 remains unclear using rodent models, particularly because one major difference between the human and murine immune system is that IL-8 is not expressed by the latter (19). In humans, IL-8 is synthesized instantaneously at local sites of infections by a variety of tissue cells e.g. endothelial cells or fibroblasts. High molecular levels of IL-8 have been detected in inflamed human pulps and chronic apical lesions immunohistochemically (9) and in periapical exudate of teeth with symptomatic and asymptomatic apical periodontitis engaging molecular methods (20). Collectively, these earlier observations are in agreement with the results of the present study, whereby IL-8 is detected in AAP, and at particularly higher levels, compared to the healthy apical tissue in SIP.

Another crucial biological process of apical periodontitis, namely bone resorption, was also evaluated in the periapical tissue fluid samples of this study. This was achieved by measuring the presence and levels of the RANKL-OPG system, which is of relevance to pulpal and periapical pathosis, particularly by initiating the molecular events that lead to osteoclastogenesis (14). However, OPG could be detected in 17% of the samples. One possible explanation for this observation could be, that the major source for OPG is connective tissue (13), which is not that prominent in the tight periapical region, especially once the dental pulp has been removed, as was in the present case. Nevertheless, molecular mechanisms of bone resorption could still be assessed by the presence of RANKL in the periapical tissue fluid. RANKL was detectable in both groups and was significantly higher expressed in cases diagnosed with SIP compared to AAP. Some studies reported significantly more RANKL-positive cells (21) and RANKL mRNA expression (22) for chronic apical lesions compared to their controls. However, healthy apical periodontal ligament cells scraped of teeth extracted for orthodontic reasons frequently served as negative controls, which is not optimal for comparison with cells from the

periapical region. Accordingly, a limitation of the present study was that due to ethical considerations it is not possible to collect control samples from healthy teeth. Yet, the presence of RANKL at higher levels in the apical tissue fluid from teeth with SIP compared to AAP confirmed in this study, indicates a higher regulation of bone resorption signaling events at the early, rather than the established, stages of apical periodontitis.

## **5. Conclusions**

In conclusion, the early periapical presence of RANKL in teeth with SIP, along with the higher presence of IL-8 at the later stage of AAP, as well as the negative correlation between these two measures indicates a reverse cascade of molecular events according to the stage of the apical periodontitis. At early stages, there is an activation of bone resorption to allow the expansion of the inflammation to the periapical region. At later stages, when the bone lesion has occurred to accommodate the inflammatory infiltrate, there is instead active chemotactic signaling that may contribute to the chronicity of periapical inflammation. These molecular characteristics of periapical pathologies should be taken into consideration, as markers for monitoring the different stages of apical periodontitis, or as potential targets for adjunctive therapeutic treatment.

## **6. Acknowledgments**

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